Research article

Toxoplasma gondii infection and peripheral-blood gene expression profiling of older people reveals dysregulation of cytokines and identifies hub genes as potential therapeutic targets

Carlos A. Naranjo-Galvis [a],[*], Kelly Y. Cardona-Londoño [a], Mary Orrego-Cardozo [a], Xabier Elcoroaristizabal-Martín [b]

[a] *Facultad de Salud, Universidad Autónoma de Manizales, Antigua Estación Del Ferrocarril, Manizales, Caldas, Colombia*
[b] *Genetracer Biotech, Santander, Spain*

ARTICLE INFO

ABSTRACT

Infections of humans with the protozoan parasite *Toxoplasma gondii* (*T. gondii*) can lead to the disease's development, even in an asymptomatic status. However, the mechanisms that result in these clinical outcomes after infection are poorly understood. This study aimed to explore the molecular pathogenesis of toxoplasmosis-related inflammation through next-generation sequencing, to assess RNA expression profiles in peripheral blood from 5 female patients with chronic toxoplasmosis and 5 healthy female controls. All plasma samples were analyzed for anti-Toxoplasma IgG and IgM antibody titers by using electrochemiluminescence. Detection of acute and chronic toxoplasmosis was carried out using the ELISA IgG avidity. We evaluated the levels of *INF-γ*, *IL-2*, *IL-12*, *TNF-α*, *IL-10*, and *IL-1β* in culture supernatants of Peripheral Blood Mononuclear Cells infected with Toxoplasma lysate antigen (TLA) prepared with tachyzoites of strain *T. gondii* RH. Differential expression analysis was performed using DESeq2, pathway and enrichment analysis of DEGs was done on WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) and Protein-protein interaction was carried out using NetworkAnalyst with STRING. In older people with chronic asymptomatic infection, a significant difference in the levels of inflammatory cytokines *INF-γ* and *IL-2* was observed compared to seronegative individuals. Our results revealed differences in the regulation of critical biological processes involved in host responses to chronic *T. gondii* infection. Gene ontology analysis revealed several biologically relevant inflammatory and immune-related pathways.

1. Introduction

Aging is generally a complex process characterized by a gradual deterioration of biological processes, affecting the immune system and central nervous system [1]. Although aging is a potent risk factor for many diseases, are mostly unknown molecular mechanisms that lead to susceptibility [2]. Aging has proven challenging to dissect in part due to its interactions with environmental influences, other genetic factors, and a large number of age-related diseases [3]. This immune aging, important in current clinical practice, is known as immunosenescence. Understanding these subtle biological changes provide us with tools to carry out suitable immunotherapy in the clinical field [4].

Among the main causes of morbidity and mortality in the elderly population are infectious diseases, and precisely a key microorganism contributing to a chronic state of host immune activity (inflamm-aging)

and neuroinflammation in humans is *T. gondii*, with the ability to alter behavior and personality among other. [5]. Latent toxoplasmosis is a prevalent chronic parasitic infection, with the ability to alter the host's behavior, due to the parasite's ability to induce neurochemical changes, cytokines release such as tumor necrosis factor-α (*TNF-α*), interleukin-12 (*IL-12*), interferon-gamma (*IFN-γ*) and *IL-1 β* or neuronal cell death [6].

The effect of parasitism on host behavior has been demonstrated between healthy immunocompetent populations, and *T. gondii* seropositivity including personality changes, suicide, schizophrenia, depression, and in very preliminary investigations, Alzheimer's disease (AD). One hypothesis suggests that parasite evolution is a sophisticated host manipulation product rather than the parasite's physiological activities. However, there is no characterization of the effects of aging on the seropositive older population, especially the age-related changes of the immune system. The current study aimed to determine which genes and
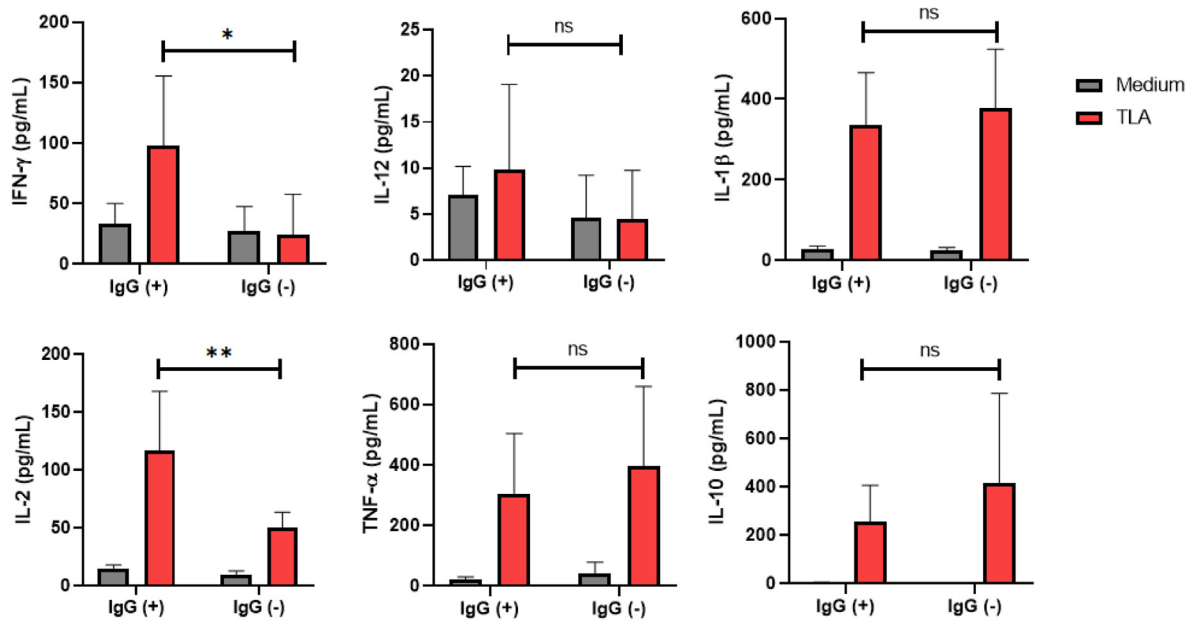
Figure 1. Comparison of cytokine production by PBMCs infected with TLA of *T. gondii* in culture supernatants at 24 h *p < 0.05, **p < 0.01; Mann-Whitney U test. IgG (+) *T. gondii* seropositive individuals; IgG (-) *T. gondii* seronegative individuals.

pathways show differential expression with age in older population blood, which could provide the basis for understanding the complex heterogeneity in toxoplasmosis in older people. We used Ion AmpliSeq Transcriptome technology and performed functional enrichment analysis for these DEGs and evaluated *INF-γ, IL-2, IL-12, TNF- α, IL-10*, and *IL-1β* levels in supernatants from Peripheral Blood Mononuclear Cells cultures infected with Toxoplasma lysate antigen (TLA) prepared with tachyzoites of the *T. gondii* RH. This study's collective data may represent a valuable resource for further advances in immunological research in the older population. Moreover, understanding the older population's immune system's principles is vital for developing vaccines that can elicit protective immunity [7].

2. Materials and methods

2.1. Samples

The Universidad Autónoma de Manizales's ethics committee approved the study protocol. The study was conducted on 10 subjects (females) over 70 years old: 5 patients with chronic toxoplasmosis and 5 healthy controls. Subjects were recruited through an open call; the exclusion criteria were: history of psychiatric or neurological disorders, allergic disease, systemic inflammatory disease or unstable medical conditions, or those who consumed systemic corticosteroids at least one

month before the test. After obtaining written informed consent, we collected blood samples from all the participants according to the Manizales city Ministry of Health's biosafety regulations and guidelines. Blood samples were collected in predetermined daytime hours between 8:00 a.m. and 9:00 a.m. to avoid the effect of diurnal variation.

2.2. Biological samples and diagnostic assays for toxoplasmosis

The participants were classified as either Toxoplasma-positive or –negative based on the concentration of specific IgM and IgG anti-*Toxoplasma gondii* antibodies in their plasma. All plasma samples were analyzed using Electro-chemiluminescence immunoassay (ECLIA) by Cobas modular platform (Cobas e 411 analyzer). Both tests (IgG and IgM) were performed by the device automatically according to the manufacturer's setting. Samples with IgM Ab titer <0.8 IU/mL were regarded as negative, 0.81–1.0 IU/mL as borderline, and >1.0 IU/mL as positive results. Samples with IgG Ab titer <0.9 IU/mL were regarded as negative, 1.0–3.0 IU/mL as borderline, and >3.0 IU/mL as positive results. Detection of acute and chronic toxoplasmosis was carried out using the ELISA IgG avidity. Using the ELISA IgG avidity method, it was observed that all IgG positive cases had chronic toxoplasmosis. A low *T. gondii* IgG avidity index shows a probable recent infection (less than 4 months) whereas a high avidity for this parasite rules out a possibility for recent infection.

Table 1. Cytokine secretion by PBMCs infected with the TLA of *T. gondii* in patients IgG (+) and IgG (-).

Cytokine[a]	Patients IgG (+)[b] Medium	Controls IgG (-)[c] Medium	P-value[d]	Patients IgG (+) [b] TLA	Controls IgG (-) [c] TLA	P-value[d]
INF-γ	33.31 ± 7.51	27.65 ± 8.86	0.640	97.99 ± 25.64	23.75 ± 15.20	0.038
IL-2	14.37 ± 1.61	9.00 ± 1.71	0.06	116.37 ± 23.15	50.07 ± 6.04	0.0079
IL-12	7.06 ± 1.39	4.58 ± 2.07	0.352	32.02 ± 22.47	4.53 ± 2.34	0.259
TNF-α	20.81 ± 4.03	41.24 ± 16.65	0.267	305.44 ± 88.86	394.04 ± 11.88	0.567
IL-10	3.90 ± 0.97	2.55 ± 1.05	0.376	254.02 ± 67.39	416.17 ± 16.54	0.392
IL-1β	27.98 ± 3.26	24.23 ± 3.31	0.444	335.44 ± 58.36	376.71 ± 65.84	0.652

[a] Levels (pg/mL).
[b] n = 5.
[c] n = 5.
[d] Results are represented as Mean ± SD; p < 0.05, Mann-Whitney U test.

Table 2. Gene ontology enrichment analysis of up-regulated differentially expressed genes associated with chronic toxoplasmosis in older people.

Category	Term	Description	Count	Genes	P-Value
GO Biological Processes	GO:0006955	Immune response	18	*IRAK3, IRF1, MCEMP1, PARP9, PPP1R14B, PRKCD, SLC11A1, TRIM25, BCL6, C3AR1, CD68, CEBPB, CLEC4D, CYBB, FCGR1A, FLOT2, GCA, IL1R2*	1.3707 e-8
GO Biological Processes	GO:0002252	Immune effector process	14	*BCL6, C3AR1, CD68, CLEC4D, CYBB, FCGR1A, GCA, IRAK3, IRF1, MCEMP1, PARP9, PRKCD, SLC11A1, TRIM25*	4.1845e-8
GO Biological Processes	GO:0006952	Defense response	15	*PARP9, PPP1R14B, PRKCD, SLC11A1, TRIM25, BCL6, C3AR1, CEBPB, CLEC4D, CYBB, FCGR1A, FLOT2, IL1R2, IRAK3, IRF1*	2.0243e-7
GO Biological Processes	GO:0045087	Innate immune response	11	*TRIM25, CLEC4D, CYBB, FCGR1A, FLOT2, IRAK3, IRF1, PARP9, PPP1R14B, PRKCD, SLC11A1*	8.0621e-7
GO Biological Processes	GO:0001816	Cytokine production	10	*BCL6, C3AR1, CEBPB, CYBB, IL1R2, IRAK3, IRF1, PRKCD, SLC11A1, TRIM25*	1.7609e-6
GO Biological Processes	GO:0060333	Interferon-gamma-mediated signaling pathway	5	*FCGR1A, IRF1, PARP9, PRKCD, TRIM25*	1.0812e-6
GO Biological Processes	GO:0031347	Regulation of defense response	10	*BCL6, C3AR1, CEBPB, CLEC4D, FLOT2, IL1R2, IRAK3, IRF1, PARP9, PRKCD*	1.5299e-6
GO Biological Processes	GO:0034341	Response to interferon-gamma	6	*FCGR1A, IRF1, PARP9, PRKCD, SLC11A1, TRIM25*	3.1586e-6
GO Biological Processes	GO:0045321	Leukocyte activation	12	*PRKCD, SLC11A1, BCL6, C3AR1, CD68, CEBPB, CLEC4D, CYBB, FLOT2, GCA, IRF1, MCEMP1*	3.9474e-6
GO Biological Processes	GO:0001817	Regulation of cytokine production	9	*BCL6, C3AR1, CEBPB, CYBB, IL1R2, IRAK3, IRF1, SLC11A1, TRIM25*	6.5352e-6

2.3. Preparation of Toxoplasma gondii lysate antigen (TLA)

Toxoplasma lysate antigen (TLA) was prepared with tachyzoites of the described *T. gondii* RH strain, after making minor changes [8]. The tachyzoites were repeatedly frozen and thawed to lyse the parasite cells in liquid nitrogen for four times and sonication (8 cycles at 20 W for 20 s). The resulting protein extract was centrifuged for 10 min at 3000 g and 4 °C to remove the cell debris, the supernatant containing native *T. gondii* antigens (TLA) was stored at -80 °C and used as antigen in the in vitro assays. Before use, they were reconstituted to 1 mg/ml in PBS.

2.4. In vitro lymphocyte proliferation assays

The PBMCs isolated from Toxoplasma-seronegative and Toxoplasma-seropositive donors were counted, and cell viability was determined by trypan blue. PBMCs were isolated from heparin-treated peripheral blood samples by Fycoll gradient. To determine the optimal concentration, they were cultivated for three days at 37 °C with 5% CO2. They were then cultured in 96-well culture plates at a density of 1×105 cells per well in 0.2 ml of RPMI-1640 supplemented with antibiotics and 10% human AB serum in the presence or absence of 10 μg/ml of concanavalin and 10 μg/ml of TLA. 5 wells per sample were used in duplicate, 1 well as control (-).

2.5. Cytokine measurement

The production of *INF-γ*, *IL-2*, *IL-12*, *TNF-α*, *IL-10*, and *IL-1β* was quantified in supernatants from PBMC previously stimulated for 72 h with TLA or medium alone. Cytokine production was measured in duplicate using the Human Cytokine Magnetic Bead Panel (MILLIPLEX MAP) according to the manufacturer's protocol (EMD Millipore, MA, USA). Before analysis, samples were centrifuged at 3000 rpm for 15 min at 4C. Detection was then performed using the multiplex technology of Luminex (MAGPIX).

2.6. RNA isolation

Total RNA was isolated from white blood cells from cases and controls (N = 10) at Genetracer Biotech Lab using the Tempus ™ Spin RNA Isolation Kit (ThermoFisher Scientific, USA) following manufacturer's instruction. RNA quality was measured using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, Calif.).

2.7. Library preparation and amplicon sequencing

Libraries were prepared from 40 ng of total RNA processed using the AmpliSeq Transcriptome Kit v2 (ThermoFisher Scientific, USA) combined with barcodes Ion Xpress™ 01–16 Kit following the manufacturer's protocol. RNA sequencing libraries were validated using the Ion Library TaqMan™ Quantification Kit (ThermoFisher Scientific, USA) in a final volume of 10ul. A reaction mixture was prepared with library concentration of each sample, mixed with the Ion Spheres Particles (ISPs) and the rest of the reagents required for the emulsion PCR. Next, the chip was loaded with the ISPs in the automated Ion Chef system (Thermo Fisher Scientific, USA) using the Ion PI™ Hi-Q™ Chef Kit and the Ion PI™ chips v3 (ThermoFisher Scientific, USA). Then, sequencing was performed on the Ion Proton sequencer (Thermo Fisher Scientific, USA), which generated more than 80 million 150bp single end reads. Finally, Raw

Table 3. Enrichment analysis result of diseases and drugs for the identified up-regulated differentially expressed genes.

Disease Enrichment	description	P value	Drug Enrichment	Description	P value
PA444614	Infection	1.2534e-8	PA164712458	Antiinflammatory agents	4.6583e-6
PA444620	Inflammation	6.0664e-7	PA164712447	Antiinfectives	9.7563e-6
PA447162	Spondylarthritis	2.4415e-6	PA452347	Glucocorticoids	1.5139e-5
PA444602	Immune system diseases	1.1127e-5	PA452174	Antivirals	2.0080e-5
PA445723	Spondylitis	1.6932e-5	PA451999	Interferons	6.6049e-5
PA166048719	Gram-positive bacterial infections	1.8858e-5	PA164712462	Antiinflammatory agents, non-steroids	1.3995e-4
PA447163	Spondylarthropathies	5.6891e-5	PA164712839	Interleukins	7.4562e-4
PA446038	Virus diseases	7.5408e-5	PA10832	Corticosteroids	8,2240e-4
PA166048822	Actinomycetales infections	1.3979e-4	PA164713378	Vaccines	8.5288e-4
PA445526	Respiratory tract infections	1.5201e-4	PA164713047	Other antivirals	9.9999e-4

Table 4. Gene ontology enrichment analysis of down-regulated differentially expressed genes associated with chronic toxoplasmosis in older people.

Category	Term	Description	Count	Genes	P-Value
GO Biological Processes	GO:0030098	Lymphocyte differentiation	6	*CAMK4, CCR7, FCRL3, ITM2A, LEF1, RHOH*	6.6925e-6
GO Biological Processes	GO:0046649	Lymphocyte activation	7	*CAMK4, CCR7, FCRL3, ITM2A, LEF1, RHOH, SIRPG*	2,4740 e-5
GO Biological Processes	GO:0002521	Leukocyte differentiation	6	*CAMK4, CCR7, FCRL3, ITM2A, LEF1, RHOH*	5.7465e-5
GO Biological Processes	GO:0009125	Nucleoside monophosphate catabolic process	2	*DNPH1, NT5E*	1.3002e-4
GO Biological Processes	GO:0002577	Regulation of antigen processing and presentation	2	*CCR7, HLA-DOB*	2.3558e-4
GO Biological Processes	GO:0030217	T cell differentiation	4	*CAMK4, CCR7, LEF1, RHOH*	3.0223e-4
GO Biological Processes	GO:0042110	T cell activation	5	*CAMK4, CCR7, LEF1, RHOH, SIRPG*	3.9816e-4
GO Biological Processes	GO:0030097	Hemopoiesis	6	*CAMK4, CCR7, FCRL3, ITM2A, LEF1, RHOH*	7.2340e-4
GO Biological Processes	GO:0048534	Hematopoietic or lymphoid organ development	6	*CAMK4, CCR7, FCRL3, ITM2A, LEF1, RHOH*	9.4902e-4
GO Biological Processes	GO:0045321	Leukocyte activation	7	*CAMK4, CCR7, FCRL3, ITM2A, LEF1, RHOH, SIRPG*	6.5352e-6

Table 5. Enrichment analysis result of diseases an drugs for the identified differentially expressed genes.

Disease Enrichment	Description	P-Value	Drug Enrichment	Description	P-Value
PA446156	Leukemia, B-Cell	9.5390e-8	PA451852	vanillin	0.010408
PA446169	Leukemia, Lymphocytic, Chronic, B-Cell	1.1057e-7	PA164712369	Angiotensin II antagonists and diuretics	0.011702
PA444756	Leukemia, Lymphoid	0.0000025048	PA448641	bisoprolol	0.011702
PA165108377	Lymphoid leukemia NOS	0.0000025048	PA10892	gliclazide	0.011702
PA444602	Immune System Diseases	0.000029889	PA164712817	Immunoglobulins	0.011931
PA446727	Lymphoma, B-Cell, Marginal Zone	0.000050652	PA164754884	immune globulin	0.011931
PA444833	Lymphatic Diseases	0.00012698	PA164713118	Other potassium-sparing agents	0.012994
PA444849	Lymphoproliferative Disorders	0.00014607	PA164749409	drospirenone	0.012994
PA153627493	Autoimmune Thyroid Disease	0.00030827	PA164743961	fludrocortisone	0.012994
PA444755	Leukemia, Hairy Cell	0.00074118	PA450061	iohexol	0.012994

data were analyzed with the Torrent Suite 5.0.2 (Thermo Fisher Scientific, USA) to generate the sequence of reads, eliminate bar codes, and low-quality reads. FASTQ files were generated using the File Exporter 4.6.0.0 plugin (Thermo Fisher Scientific, USA).

2.8. Gene expression analysis

AmpliSeq data were analyzed and adjusted by quality using the tool SAMStat v1.5 [9]. Later, cleaned reads were aligned onto the human reference genome UCSC Hg19 (TDB, 2014) using the Ion Torrent Mapping Alignment Program (TMAP). Follow by estimation of abundances with BED Tools v 2.26 [10]. The DeSeq2 v.1.12.4 R package [11] was used to perform differential expression analysis of sequenced data. First, genes were filtered based on reading coverage, and only genes that had average coverage of at least 50 reads across all samples were retained. P-values were corrected for significance and the Benjamini-Hochberg correction was applied to estimate the false discovery rate (FDR). The value of FDR ≤ 0.1, $|log2FC| > 0.5$ and corrected p-value ≤ 0.05 were used as the cutoff point for statistical significance.

2.9. Gene ontology (GO) enrichment analysis of differentially expressed genes

The detected DEGs were subjected to functional enrichment analyses using WEB-based Gene SeT AnaLysis Toolkit (WebGestalt, http://www.webgestalt.org/), an online software toolkit comprising information from various public resources for biological analysis [12]. The enrichment analysis such pathways, diseases, and drugs were carried out with top 10 results as significant using a hypergeometric test and Benjamini & Hochberg method.

2.10. Protein-protein interaction (PPI) network of the (DEGs)

The protein-protein interaction (PPI) network of the (DEGs) encoding proteins was analyzed, and the first-order network was performed using Network Analyst with STRING interactome database [13] at the confidence score cutoff 900.

2.11. In silico analysis

To understand the potential association between miRNAs and differentially expressed mRNAs obtained in the study, the differentially expressed mRNAs were imported into the miRWalk algorithm (version 3.0, http://mirwalk.umm.uni-heidelberg.de/) [14] based on six bioinformatics algorithms (miRanda, DIANAmT, PicTar, miRDB, TargetScan, and miRWalk) to predict their target miRNAs. The co-predicted target miRNAs by three or more mentioned software were considered putative targets for subsequent analysis. For designing the Gene-miRNA Interaction network, the web-based application NetworkAnalyst was used.

Table 6. Reactome pathway analysis of up-regulated differentially expressed genes associated with chronic toxoplasmosis in older people.

Gene Set	Description	P-Value	FDR
R-HSA-168256	Immune System	2.7866e-6	0.0036058
R-HSA-168249	Innate Immune System	5.1225e-6	0.0036058
R-HSA-877300	Interferon-gamma signaling	7.5429e-6	0.0036058
R-HSA-6798695	Neutrophil degranulation	8.3468e-6	0.0036058
R-HSA-913531	Interferon Signaling	2.8781e-4	0.099468
R-HSA-1280215	Cytokine Signaling in the Immune system	8.1074e-4	0.23349
R-HSA-2029485	Role of phospholipids in phagocytosis	2.5581e-3	0.63150
R-HSA-1236975	Antigen processing-Cross presentation	3.2600e-3	0.70416
R-HSA-1222556	ROS, RNS production in phagocytes	4.1739e-3	0.80139
R-HSA-3371497	HSP90 chaperone cycle for steroid hormone receptors (SHR)	1.1146e-2	1

Table 7. Reactome pathway analysis of Down-regulated differentially expressed genes associated with chronic toxoplasmosis in older people.

Gene Set	Description	P-Value	FDR
R-HSA-74259	Purine catabolism	3.6298e-4	0.37597
R-HSA-15869	Metabolism of nucleotides	4.3515e-4	0.37597
R-HSA-8956319	Nucleobase catabolism	1.3914e-3	0.80144
R-HSA-111932	CaMK IV-mediated phosphorylation of CREB	7.5585e-3	1
R-HSA-9022535	Loss of phosphorylation of MECP2 at T308	7.5585e-3	1
R-HSA-442717	CREB phosphorylation through the activation of CaMKK	9.0638e-3	1
R-HSA-5358493	Synthesis of diphthamide-EEF2	1.2068e-2	1
R-HSA-4411364	Binding of TCF/LEF: CTNNB1 to target gene promoters	1.2068e-2	1
R-HSA-8951430	RUNX3 regulates WNT signaling	1.2068e-2	1
R-HSA-4641265	Repression of WNT target genes	1.8051e-2	1

The miRmap software [15] addresses the challenges in post-transcriptional repression of miRNAs in the human genome by probabilistic thermodynamic, evolutionary, and sequence-based features. In miRmap, the seed pairing of miRNA with the mRNA of the corresponding gene was analyzed.

Gene regulatory events play a crucial role in several developmental and physiological processes in a cell. Macromolecules such as proteins, RNA, and genes work in coordination to generate operative responses under different conditions [16]. The RegNetworks [17] is a database that contains types (Transcription Factor-Gene, Transcription Factor-Transcription factor, microRNA-Transcription Factor) of transcriptional and posttranscriptional regulatory relationships for mouse and human. The conservation of knowledge about the binding site of transcription factor (TFBS) can also be implemented to couple the potential regulation between targets and their regulators. For designing the TF-Gene Interaction network, the web-based application NetworkAnalyst was used.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software. The Kolmogorov-Smirnov test was applied for assessing the normality of the data distribution. The concentration of each cytokine was expressed in pg/mL. Cytokines levels among the two groups were compared using t-test and Mann-Whitney U test. Differences with a two-tailed value of $p < 0.05$ were considered as statistically significant.

3. Results

The Ethics Committee of the Universidad Autónoma de Manizales approved this study's protocol [project 515–075]. A total of 10 blood donors participated in this study. RNA was isolated from whole blood from 5 patients with *T. gondii* seropositive (mean age ±standard deviation [SD], 75.37 ± 17.27 years) and 5 healthy controls (mean age ±standard deviation [SD], 74.29 ± 13.81 years). All participants were females.

3.1. Cytokines production by PBMCs infected with TLA of T. gondii

We first investigated the cytokines secreted by PBMCs obtained from *T. gondii*-seropositive elderly people and 5 healthy controls, after stimulation with Toxoplasma lysate antigen (TLA) prepared with tachyzoites of the RH strain of *T. gondii*. In response to the infection with *T. gondii*, the
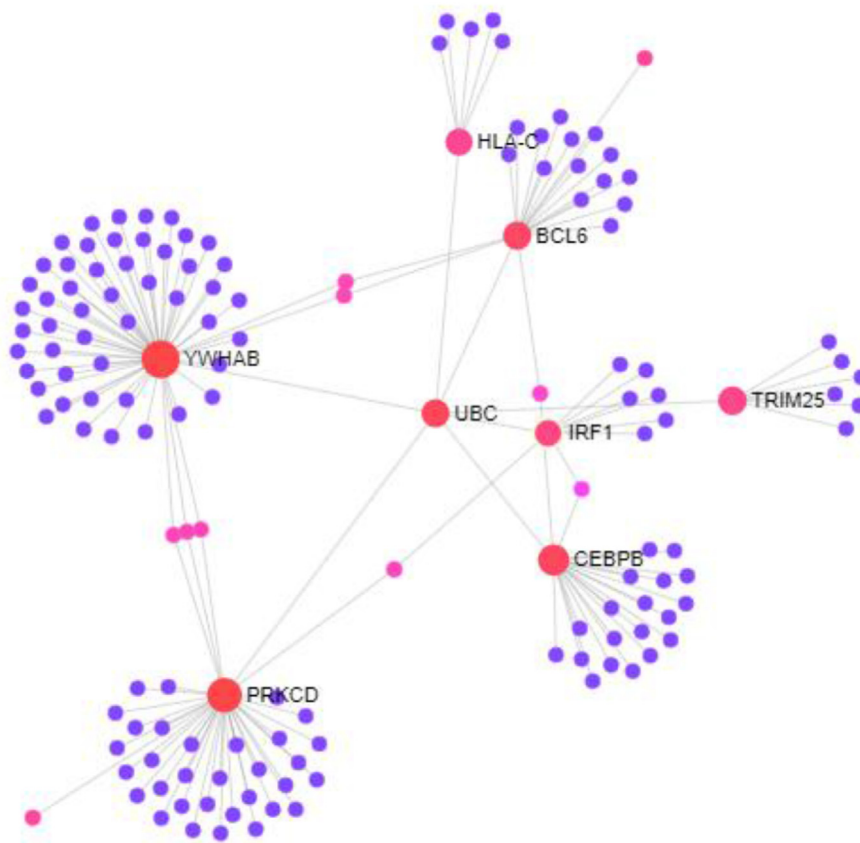

Figure 2. PPI network of hub genes (up-regulated) of older people with chronic toxoplasmosis. The nodes represent proteins, and edges represent connections/interactions. The gradual color represents the degree. *LEF1, TRAF4, CAMK4, CREBBP*, and *BIRC3* in DEGs down-regulated (Figure 3).
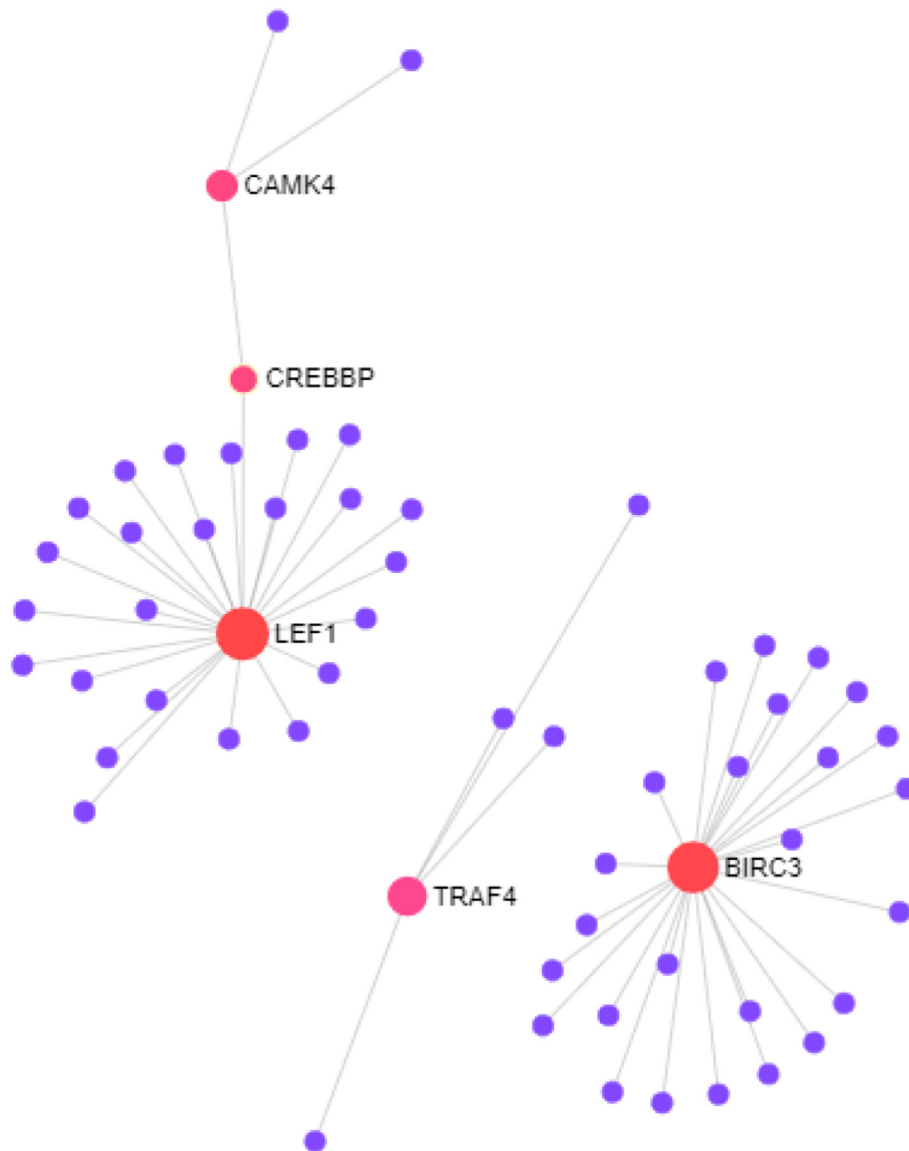
Figure 3. PPI network of hub genes (down-regulated) of older people with chronic toxoplasmosis. The nodes represent proteins, and edges represent connections/interactions. The gradual color represents the degree.

host sets up an immune reaction, mainly of cellular type, via T lymphocytes—essentially helper T lymphocytes (Th1), characterized by pro-inflammatory cytokine production such as interferon-gamma (*IFN-γ*), interleukins 2 and 12 (*IL-2* and *IL-12*) and tumor necrosis factor (*TNF-α*) [18]. We first investigated the cytokines secreted by the PBMCs obtained from older people *T. gondii* seropositive and 5 healthy controls, upon stimulation with Toxoplasma lysate antigen (TLA) prepared with tachyzoites of the *T. gondii* RH strain. *INF-γ* production and *IL-2* after TLA infection was higher in PBMCs from the seropositive individual's group than in the seronegative individual's group (P = 0.038 and P = 0.0079, respectively), indicating that these cytokines tended to be higher in seropositive older people. It is well established that *IFN-γ* is important to promote Th1-directed adaptive immune responses and to control parasite replication during chronic infection via *IFN-γ* mediated NO synthesis in different host cells such as macrophages. Although the principal cytokine controlling this infection is *IFN-γ*, other cytokines have been implicated [19]. *IL-2* is an important growth factor involved in the activation and expansion of T-cell responses during an immune response. Since production of *IFN-γ* by T cells is the major mediator of resistance to *T. gondii*, *IL-2* have been demonstrated to be involved in the optimal production of

IFN-γ by T cells [20]. Contrarily, *IL-10* inhibits the killing of *T. gondii* by human macrophages and leads to suppression of cell-mediated immunity in response to this parasite and decreases the pathology linked to its infection [21] (Figure 1).

In contrast, the results indicate that, under experimental conditions used, no significant differences were observed for *IL-12*, *TNF-α*, *IL-10*, and *IL-1β* secretion among groups. The monitoring of the immune response can provide valuable information that can help in understanding the mechanisms of immune system control over parasites (Table 1).

3.2. Differential expression analysis and gene ontology enrichment

Amplicon RNA sequencing from peripheral blood of subjects generated an average of ~12 million reads per sample, of which more than 94% aligned to the reference genome, data analysis identified 13227 annotated amplicons genes as expressed in the sequenced samples. DESeq2 identified a total of 75 genes as differentially expressed (p-value ≤ 0.05 after FDR correction) between cases and controls, of which 46 were up-regulated, and 29 were down-regulated (Table S1). GO enrichment analysis was performed to obtain insights into the biological roles
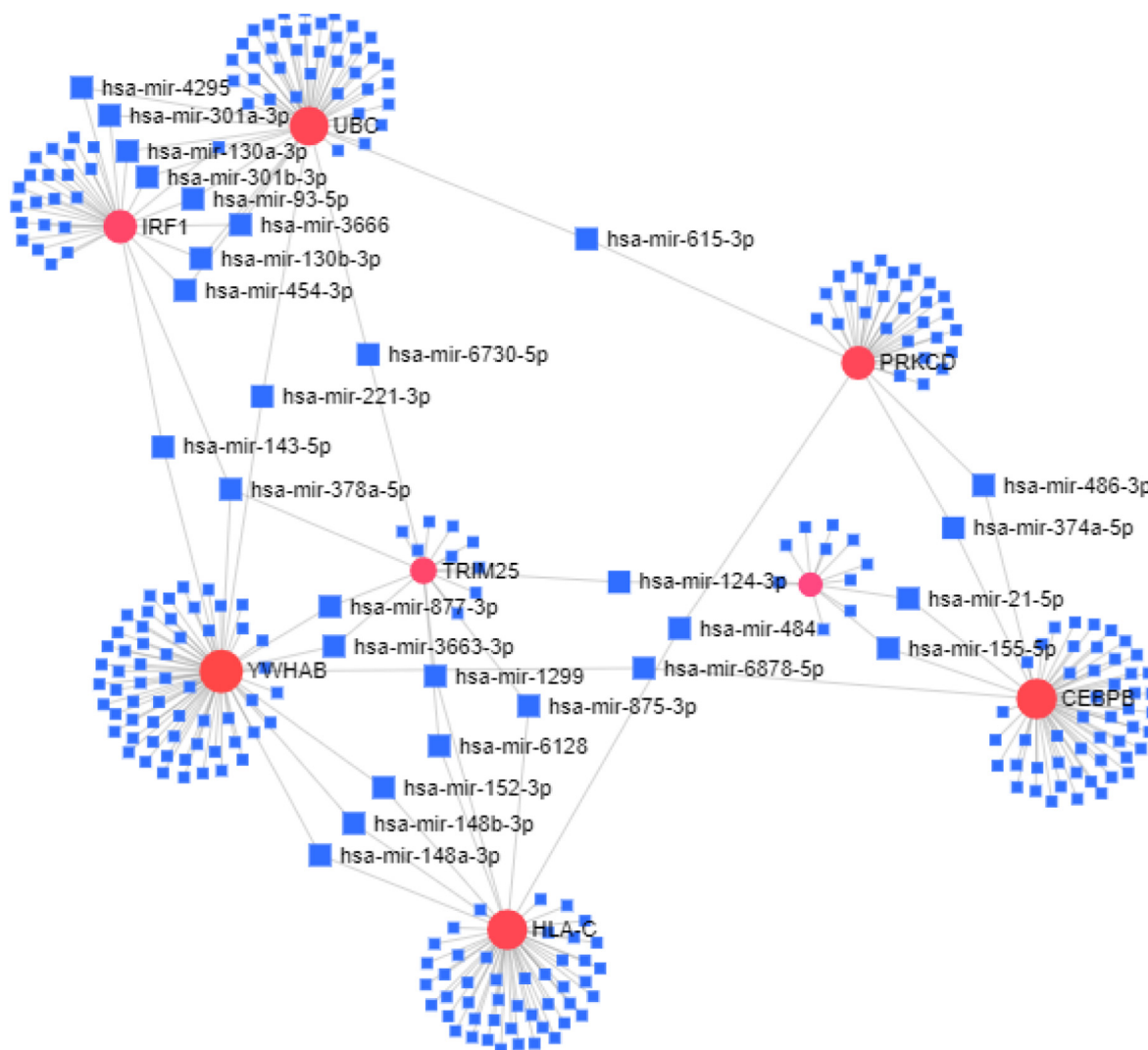
Figure 4. A PPI network for eight common responsible genes. The network consists of 295 nodes and 317 edges, and eight seed nodes. Nodes are proteins, and the edges establish a relationship between proteins.

of DEGs. Only the annotated DEGs were selected and tested against the background set of all genes with GO annotation. GO categories were examined separately.

We found that for up-regulated genes, almost all of the significantly enriched GO terms in the biological process category were associated with the immune system, such as immune response (GO:0006955), immune effector process (GO: 0002252), defense response (GO: 0006952), Innate immune response (GO:0045087) and Cytokine production (GO:0001816). The overview of GO analysis results for the DEG with adjusted p-value with the number of genes involved is summarized in (Table 2).

Emerging transcriptomic approaches can help decipher how aging interventions prioritize which transcripts to regulate and, in general, how the aging brain maintains this decreased translation rate without compromising cellular health and integrity. Here, we focus specifically on how the parasite alters the immune response, as this is crucial to our understanding of how infection is controlled and since the host's immune response is a primary target of *T. gondii* virulence factors. Unbiased analysis of gene expression has identified new host genes associated with *T. gondii* infection, which can be validated in subsequent functional studies. We show that some parts of the host response can be used as potential biomarkers of disease, representing viable targets for drug therapy. Finally, this study is a necessary preliminary analysis to reveal

the starting points of this pathological cascade in older adults infected with *T. gondii*. By applying the wide range of recently developed tools, it is hoped that we can identify critical elements that can be addressed therapeutically, linking telltale nodes of aging with *T. gondii* infection. Results revealed that the main diseases associated with hub genes were infection, inflammation, Spondylarthritis, immune system disease, anti-inflammatory agents, antiinfectives, glucocorticoids, antivirals, and interferons are the top 5 drugs found in drug enrichment analysis (Table 3).

For down-regulated genes, the most significantly enriched biological process GO terms were lymphocyte differentiation (GO: 0030098), lymphocyte activation (GO: 0046649), and leukocyte differentiation (GO: 0002521) (Table 4).

The results provide an important collection of differential gene expression data in chronic versus healthy donors. To verify the utility of our processed gene signature, WebGestalt database was utilized for finding suitable drug molecules that may be used for treatment of chronic toxoplasmosis caused by hub genes expressed differentially. The enriched disease terms for the DEG were leukemia, B-Cell, Leukemia, Lymphocytic, Chronic, B-Cell, Leukemia, Lymphoid and Lymphoid leukemia NOS. Drug association analysis was performed for all hub gene candidates by using WebGestalt database and results obtained were depicted in Table 5. Vanillin, Angiotensin II antagonists and diuretics, bisoprolol, gliclazide and immunoglobulins are identified best drug candidates.
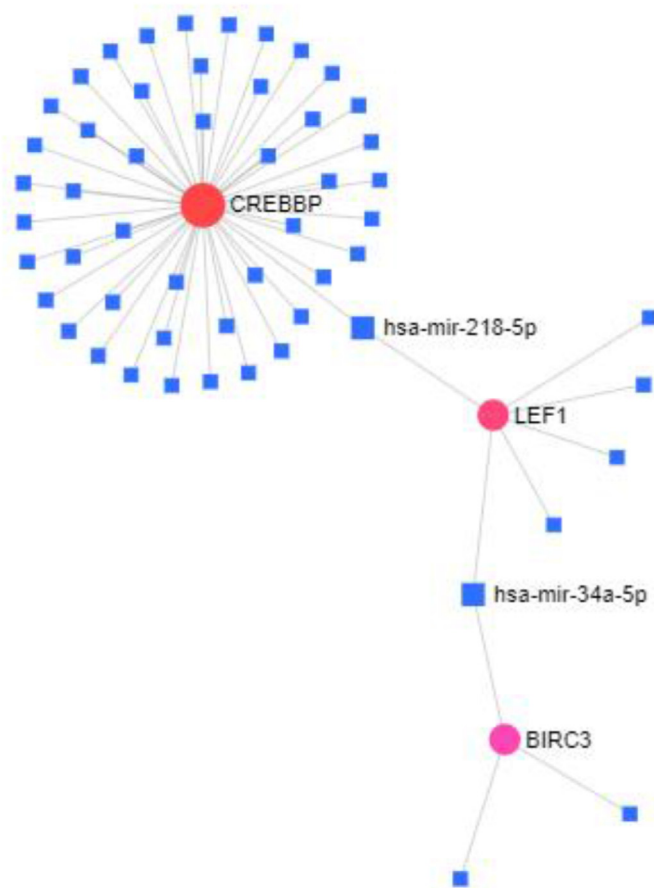
Figure 5. A PPI network for five common responsible genes. The network consists of 57 nodes and 56 edges, and three seed nodes. Nodes are proteins, and the edges establish a relationship between proteins.

3.3. Pathway enrichment analysis of differentially expressed genes

To further evaluate the biological significance of the DEGs, we also performed Reactome pathway enrichment analysis. Hypergeometric tests with a cut-off value of $p < 0.05$ were used as the criterion for pathway detection. We found that up-regulated genes were significantly enriched in 10 pathways, among which five pathways were immune-related (Table 6).

Table 7 shows down-regulated genes were significantly enriched in 10 pathways, among which three pathways were metabolism-related.

3.4. Protein-protein interaction network of differentially expressed genes

Protein-protein interaction analysis was performed on all DEGs. Since the biological processes occur in a network biology context, to provide functional insights into the identified DEGs roles, we have reconstructed the PPI network around the proteins encoded by the DEGs using the publicly available PPI database STRING via NetworkAnalyst tool. Through a topological measure degree, we have detected the presence of eight hub proteins in DEGs up-regulated: *YWHAB, PRKCD, CEBPB, BCL6, IRF1, TRIM25, UBC,* and *HLA-C* (Figure 2).

3.5. The regulatory network of differentially expressed mRNAs and miRNAs associated with chronic toxoplasmosis in older people

In miRWalk, 16 miRNAs (hsa-miR-181b-5p, hsa-miR-485–5p, hsa-miR-216b-5p, hsa-miR-504–3p, hsa-miR-4753–3p, hsa-miR-6715b-5p, hsa-miR-5011–5p, hsa-miR-203a-5p, hsa-miR-423–5p, hsa-miR-346, hsa-miR-

93–5p, hsa-miR-3666, hsa-miR-378a-5p, hsa-miR-1182, hsa-miR-651–3p, hsa-miR-4660) were associated with 8 genes up-regulated (Figure 4).

15 miRNAs (hsa-miR-649, hsa-miR-571, hsa-miR-449a, hsa-miR-300, hsa-miR-381–3p, hsa-miR-449b-5p, hsa-miR-129-1-3p, hsa-miR-17–5p, hsa-miR-29a-3p, hsa-miR-33a-5p, hsa-miR-29b-3p, hsa-miR-34a-5p, hsa-miR-4319, hsa-miR-153–3p, hsa-miR-6729–3p) were associated with 5 genes down-regulated (Figure 5).

3.6. Identification of transcription factors from RegNetworks

We identified TFs that may regulate the expression of DEGs at transcriptional levels. In RegNetworks, 80 transcription factors (*ABL1, GABPA, HDAC5, CREBBP, DAB2, EEF1A1, EGR2, EGR3, EP300, ESRRA, FOXA1, HABP4, HNF4A, HNRNPK, ATF2, ATF4, CEBPA, BACH1, ATF2, AHR, ARNT, CREB1, CTCF, EIF2AK2, ELK1, EP300, HMGA1, JUN, MAX, MYC, PPARG, TFAP2A, TFAP2C, USF1, XBP1, SPI1, SP1, SFPQ, RXRG, RXRB, RXRA, BACH, CREB1, DAZAP2, E2F1, E2F2, E2F3, E2F4, E2F5, E2F6, E2F7, EGR1, FOS, FOSB, FOSL1, HDAC5, NFKB1, NFKB2, NFYAABL1, GABPA, HDAC5, DAB2, EGR2, EP300, FOXA1, CREB1, EGR1, STAT3, MYB, STAT1, STAT5B, NFKB1, EIF2AK2, STAT4, MYC, USF1, PPARD, RXRA, RXRB, RXRG*) were associated with the 8 genes up-regulated (Figure 6).

18 transcription factors (*ELK1, NFKB1, NFKB2, RELA, TP53, CTBP1, CTNNB1, CREBBP, EP300, RELA, ATF4, ATF4, CREB1, CREB3, CREB3L1, CREB3L3, CREB3L4, CREB5*) were associated with the five genes down-regulated (Figure 7).

Up-regulated and down-regulated gene search in chronic toxoplasmosis results in 13 genes with a validated efficacy in clinical research. The miRNA search for these 13 genes in miRWalk resulted in identifying 31 miRNAs and 98 transcription factors with a maximum probability of becoming biomarkers for chronic toxoplasmosis based on an association of genes and transcription factors (Table 8).

Table 9 summarizes the hub proteins identified from the protein-protein interactions encoded by the up-differentially expressed and down-differentially genes in older people with chronic toxoplasmosis.

4. Discussion

The aging process is characterized by a continuous remodeling. The main actors are apoptosis, oxidative stress, DNA repair, immune response and inflammation. One of the most recent theories on aging focuses on the chronic low-grade inflammation named "inflammaging" [22].

A major contributor of inflammaging is the antigenic load of persistent infection, It is also clear that chronic *T. gondii* infection alters human monocytic cell phenotypes and functions across lifetimes [23], and is not immunologically silent as large quantities of pro inflammatory cytokines are produced/released and are the expression of a system that involves environment, genes and polymorphisms. Some studies have found alterations in individuals¯ behavior associated with *T. gondii* seropositivity. *T. gondii* causes latent chronic infections of worldwide concern. Its subclinical influence on behavior and cognition is not well understood [24].

The long-term host effects caused by the parasite *T. gondii* are poorly understood. Mice with chronic infection have alterations in connectivity [25], neurotransmitters [26], seizures, memory, neurobehaviors [27] and form long-lasting chronic infections in the central nervous system (CNS) of humans [28]. Some epidemiologic serologic studies show associations between seropositivity for *T. gondii* and some human neurologic diseases, including memory loss [29].

In the present study, we focused on the effects of chronic, latent Toxoplasma infection in the PBMCs from older people to produce cytokines, when cultured in the presence of Toxoplasma lysate antigen (TLA) prepared with tachyzoites of the *T. gondii* RH strain. The significant production of *IL-2* and *INF-γ* highlights the importance of these two cytokines in the immunopathology associated with toxoplasmosis. Interestingly, neither *IL-12, TNF- α, IL-10,* and *IL-1β* concentrations showed
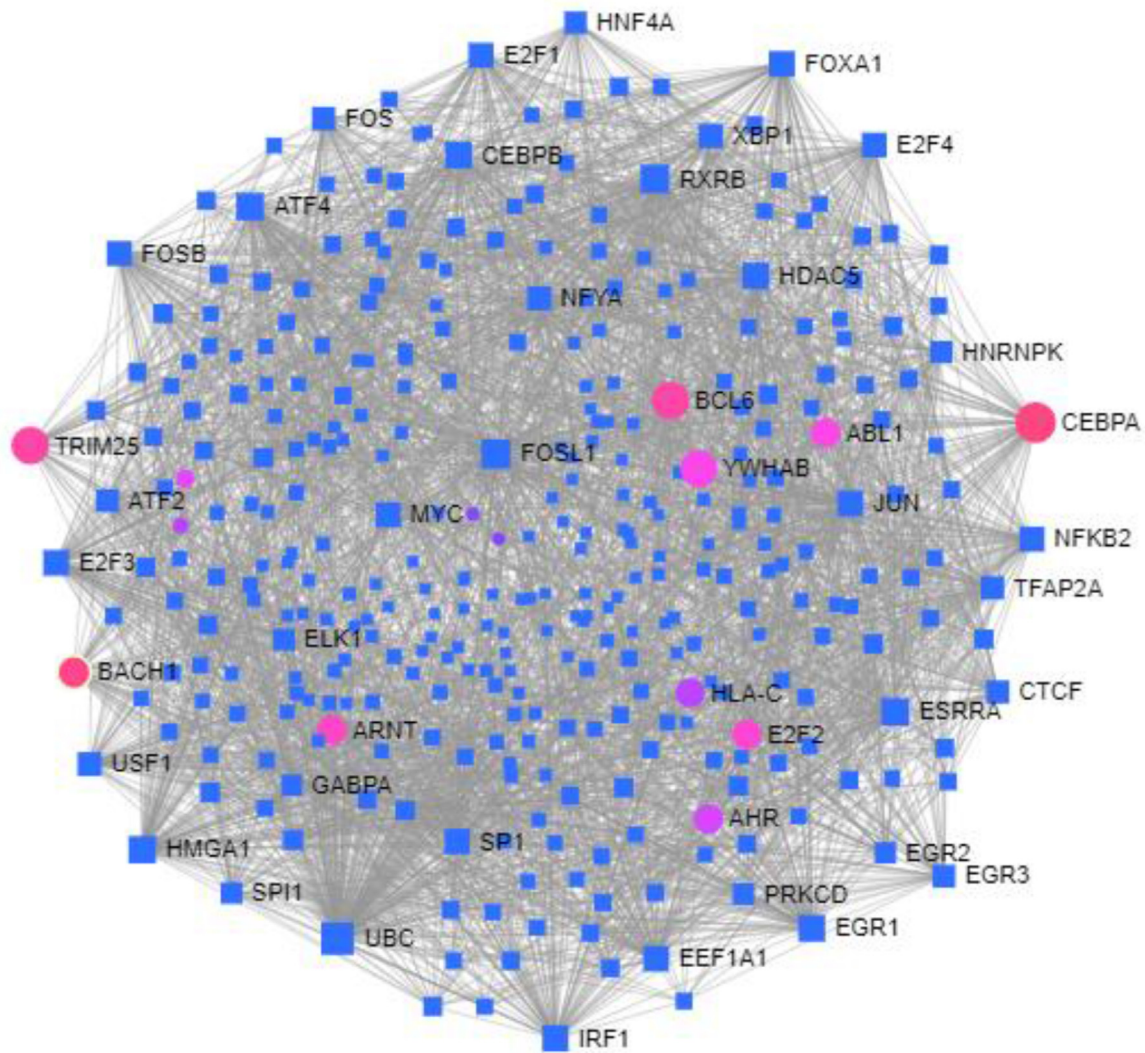
Figure 6. Analysis of Transcription Factors interaction networks based on differentially expressed genes (up-regulated) identified in chronic toxoplasmosis in the older population. The network consists of 351 nodes and 2842 edges, and 60 seed nodes.

any alterations in supernatants from Peripheral Blood Mononuclear Cells cultures infected with Toxoplasma lysate antigen (TLA).

IL-2 is considered important in mediating resistance against a variety of different intracellular parasites [30]. Kelly et al. described the *IL-2* and *INF-γ* production from peripheral blood mononuclear cells stimulated in vitro with Toxoplasma antigen from a seropositive donor [31]. To our knowledge, there are no other studies evaluating *IL-2* and *IFN-γ* production from PBMCs stimulated with Toxoplasma lysate antigen (TLA) in older people. *IL-2* is produced by T-cells and controls the expression of the transcription factors, effector cytokines and chromatin regulators. Activates NK-cells, stimulates B-cells and is necessary for the proliferation of T-lymphocytes [32].

During chronic toxoplasmosis, the production of *IFN-γ* has been shown to play an important role in the switch to bradyzoite production, as is the activation of CD8+ cytotoxic T cells and the induction of immunity-related GTPases (IRG) and is indispensable for the survival of the host [33].

Transcriptomics great promise for personalized and precision medicine and can provide a comprehensive understanding of biological processes [34]. Differential gene expression analysis is used to identify key genes that undergo changes in expression relative to healthy individuals, as well as patients with other diseases. These key genes can act as diagnostic, prognostic and predictive markers for disease. Gene expression

"signatures" in blood have the potential to be used for diagnosis of infectious diseases, where current diagnostics are unreliable, ineffective, or of limited potential. With methods more sophisticated blood transcriptome profiling may become suitable for use in clinical practice for diagnosing, predicting, and personalizing treatment for chronic toxoplasmosis.

Our study identified specific blood gene expression patterns associated with chronic toxoplasmosis when compared to healthy individuals. In our study, gene expression profiling analysis revealed the core genes and pathways associated with chronic toxoplasmosis and allowed the identification of targets for therapeutic strategy. Bioinformatics methods were applied to analyze the raw data and we identified 75 DEGs, including 46 up-regulated and 29 down-regulated DEGs. Various bioinformatics analyses such as GO, pathways, disease, drugs, and networks were utilized for the integrated approach. The enrichment analysis of these genes gave an overview of possible associated diseases and dysregulated pathways. The PPI network of DEG was constructed using the STRING database and the main genes *YWHAB*, *PRKCD*, *CEBPB*, *BCL6*, *IRF1*, *TRIM25*, *UBC* and *HLA-C* were obtained. Druggable genes (DG) are vital and can act as a therapeutic target for disease.

The *CEBPB* (Enhancer Binding Protein β-CCAAT) transcription factor regulates numerous genes involved in essential functions such as hematopoiesis, cell cycle, inflammation, and host immune responses [35], is
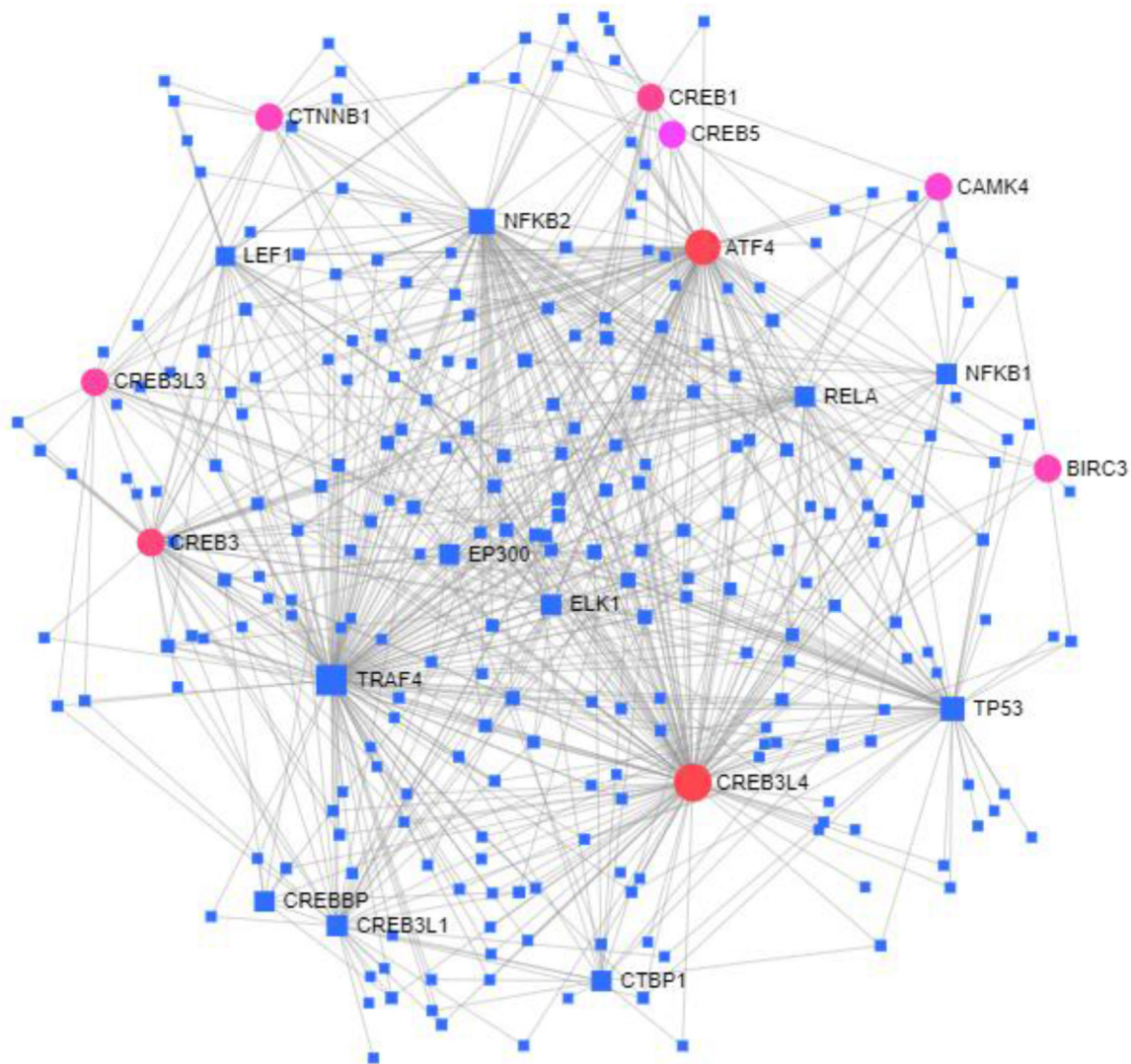
Figure 7. Analysis of Transcription Factors interaction networks based on differentially expressed genes (down-regulated) identified in chronic toxoplasmosis in the older population. The network consists of 269 nodes and 732 edges, and 20 seed nodes.

activated by multiple inflammatory stimuli, including LPS and *IL-17* [36]. Furthermore, *IL-17* activates the CCAAT/Enhancer Binding Protein family of transcription factors, particularly CEBPB [37], but the biological role of this pathway in chronic toxoplasmosis is not well defined. Cebpb−/− mice are highly susceptible to systemic Listeria monocytogenes [38]. In contrast, Cebpb−/− mice were resistant to oral candidiasis [39].

The transcriptional repressor B-cell Lymphoma 6 (*BCL6*), as a transcriptional repressor, mediates the innate immune response's counter-regulation and represses NF-κB activity [40]. Also, *BCL6* can directly repress its target proinflammatory cytokines and chemokines by specifically binding to the target gene promoters in macrophages [41]. We also found that *PRKCD* and *IRF1* were significantly overexpressed in older people with chronic toxoplasmosis. *PRKCD* is a serine-threonine kinase that encodes protein kinase C-δ and phosphorylates myristoylated alanine-rich C kinase substrate regulating differentiation apoptosis and cell cycle in several cellular models. The phosphorylation is known to induce neurite degeneration via instability of the actin network in mouse and humans brains [42]. We identified *IRF1* (interferon regulatory factor 1) itself as a transcription factor, regulated by *STAT1*, the absence of which increases susceptibility to Toxoplasma infection [43].

Formin-like protein 1 (*FMNL1*) is expressed in various tissues, such as the spleen, peripheral blood leukocytes and thymus [44]. *FMNL1*

expression is correlated with cell adhesion, phagocytosis, survival, and cell migration in macrophages [45]. On the other hand, *TRIM25* is an E3 ubiquitin ligase enzyme crucial for antiviral immune response associated with *IFN* signaling involved in various cellular processes. *TRIM25* [46] has recently been identified as an RNA-binding protein, important for its role in regulating intracellular signaling, innate immunity, cell development, and participating NF-κB signaling pathway implicating regulatory functions in inflammation and immunity [47].

GO analysis revealed genes involved in lymphocyte differentiation (GO: 0030098), lymphocyte activation (GO: 0046649), and leukocyte differentiation (GO: 0002521). The top hub genes were obtained: *BIRC3*, *LEF1*, *TRAF4*, *CAMK4*, and *CREBBP*.

Recently, studies have demonstrated a pivotal role for (miRNAs) to infection by apicomplexan parasites as well as functionality of host mature various immune cells [19] and can directly affect the expression of thousands of genes [48]. For example, miR-125b appear to strongly impact on proinflammatory cytokine production, such as *IFN-γ* and *IL-2* [49]. Additionally, there have been fewer reports the role of each mRNA/miRNA target in specific pathways during infection *T. gondii*. In this context, an increased understanding of the interactions among altered behavior, neuroinflammation, and infection may lead to new approaches for the treatment and prevention of these disorders and insight into the interaction between host and parasite.

Table 8. Genes, miRNAs & Transcription Factors associated with regulation of chronic toxoplasmosis in the older population.

Genes	miRNAs (miRWalk)	Gene-miRNA score (miRmap)	Transcription factors (RegNetworks)
YWHAB	hsa-miR-181b-5p	3.42%	ABL1, GABPA, HDAC5
	hsa-miR-485–5p	90.86%	
	hsa-miR-216b-5p	80.13%	
	hsa-miR-504–3p	Nil	
	hsa-miR-4753–3p	58.95%	
	hsa-miR-6715b-5p	90.78%	
PRKCD	hsa-miR-5011–5p	99.85%	CREBBP, DAB2, EEF1A1, EGR2, EGR3, EP300, ESRRA, FOXA1, HABP4, HNF4A, HNRNPK
	hsa-miR-203a-5p	Nil	
CEBPB	hsa-miR-423–5p	Nil	ATF2, ATF4, CEBPA
BCL6	hsa-miR-346	97.99%	BACH1, ATF2
IRF1	hsa-miR-93–5p	74.01%	AHR, ARNT, CREB1, CTCF, EIF2AK2, ELK1, EP300, HMGA1
	hsa-miR-3666	96.59%	
	hsa-miR-378a-5p	94.76%	
TRIM25	hsa-miR-1182	99.70%	JUN, MAX, MYC, PPARG, TFAP2A, TFAP2C, USF1, XBP1
UBC	hsa-miR-651–3p	Nil	SPI1, SP1, SFPQ, RXRG, RXRB, RXRA, BACH, CREB1, DAZAP2, E2F1, E2F2, E2F3, E2F4, E2F5, E2F6, E2F7, EGR1, FOS, FOSB, FOSL1, HDAC5
HLA-C	hsa-miR-4660	92.19%	NFKB1, NFKB2, NFYA
BIRC3	hsa-miR-649	Nil	ELK1, NFKB1, NFKB2, RELA, TP53
	hsa-miR-571	81.55%	
LEF1	hsa-miR-449a	92.82%	CTBP1, CTNNB1, CREBBP, EP300
	hsa-miR-300	88.35%	
	hsa-miR-381–3p	85.79%	
	hsa-miR-449b-5p	92.98%	
TRAF4	hsa-miR-129-1-3p	73.02%	NFKB1, NFKB2, RELA
	hsa-miR-17–5p	78.07%	
CAMK4	hsa-miR-29a-3p	Nil	ATF4, CREBBP, EP300
	hsa-miR-33a-5p	Nil	
	hsa-miR-29b-3p	Nil	
	hsa-miR-34a-5p	Nil	
	hsa-miR-4319	Nil	
CREBBP	hsa-miR-153–3p	84.06%	ATF4, CREB1, CREB3, CREB3L1, CREB3L3, CREB3L4, CREB5, CTNNB1
	hsa-miR-6729–3p	Nil	

These new data and analysis will provide insights extending beyond infection, to common pathways of neurodegeneration and shared upstream regulators parasites perturb [50]. Data also are accumulating that a variety of infections cause cognitive impairment. Thus, a new paradigm to integrate knowledge of pathogenesis, prevention, and treatment is needed.

Analysis of differentially expressed genes have allowed us to identify core genes and relevant immune pathways that are activated in asymptomatic older patients with chronic toxoplasmosis that could represent potential biomarkers for early diagnosis and treatment.

The study is an initial investigation with a small sample size. However, future expression studies with blood will require larger sample sizes including older men to establish the robustness of the findings.

5. Conclusions

Our results revealed that cytokine signaling responses as an inflammatory hallmark in Toxoplasma gondii-infected older people, may be associated with modulation of immune responses of Th1 CD4+ T cells. The results indicate that *T. gondii* infection of IgG positive individuals induces changes in gene expression profile in relevant immunological pathways that could predict disease trajectories.

Declarations

Author contribution statement

Carlos Andrés Naranjo-Galvis: conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data; wrote the paper.

Kelly Y Cardona-Londoño: performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data.

Mary Orrego-Cardozo: performed the experiments; contributed reagents, materials, analysis tools or data.

Xabier Elcoroaristizabal-Martín: performed the experiments; contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Table 9. The summary of the hub proteins identified.

Gene Symbol	Full Name	Feature
YWHAB	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta	Role in linking mitogenic signaling and the cell cycle machinery
PRKCD	Protein kinase C delta	Role in the regulation of various cellular processes, including cell proliferation, apoptosis, and differentiation
CEBPB	CCAAT enhancer-binding protein beta	Transcription factor regulating the expression of genes involved in immune and inflammatory responses
BCL6	BCL6 transcription repressor	Potential transcriptional regulatory activity
IRF1	Interferon regulatory factor 1	Transcriptional regulator which displays a remarkable functional diversity in the regulation of cellular responses
TRIM25	Tripartite motif-containing 25	Involved in innate immune defense
UBC	Ubiquitin C	Plays a crucial role in maintaining cellular ubiquitin levels under stressful conditions
HLA-C	Major histocompatibility complex, class I, C	Play a central role in the immune system by presenting peptides derived from endoplasmic reticulum lumen
BIRC3	baculoviral IAP repeat-containing 3	Modulates inflammatory signaling and immunity
LEF1	lymphoid enhancer-binding factor 1	Regulates T-cell receptor alpha enhancer function
TRAF4	TNF receptor-associated factor 4	Plays a role in the activation of JNK and NF-kappa-B and the regulation of apoptosis and cell survival
CAMK4	calcium/calmodulin-dependent protein kinase IV	It is involved in synaptic plasticity, neuronal transmission, and neuronal gene expression required for neuronal homeostasis and brain development.
CREBBP	CREB binding protein	Plays a critical role in the control of cell proliferation, homeostasis, and embryonic development

Declaration of interests statement

The authors declare no conflict of interest. Xabier Elcoroaristizabal Martín is employed by Genetracer Biotech and collaborates with Universidad Autónoma de Manizales. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e10576.

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